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研究課題名（和文）　破骨細胞前駆細胞プールによる骨代謝調節機構の解析

研究課題名（英文）　Analysis of the regulation of bone metabolism by the pool of osteoclast precursors

研究代表者
溝口　利英（MIZOGUCHI TOSHIHIDE）
松本歯科大学・総合歯科医学研究所・講師
研究者番号：90329475

研究成果の概要（和文）：以前我々は、細胞増殖が停止した破骨細胞前駆細胞(Cell cycle-arrested quiescent osteoclast precursors: QOP)を同定した。QOP は、細胞周期の進行を介さずに破骨細胞へ分化する。今回我々は、①QOP は血流中にも存在し、骨吸収刺激に伴い骨吸収部位へ遊走すること、②QOP は骨表面で骨芽細胞が発現する M-CSF(CSF1)依存的に RANK の発現を上昇し、この RANK の発現上昇には c-Fos が必要であることを明らかにした。

研究成果の概要（英文）：We examined the characteristics of osteoclast precursors in vivo, and found that the precursor are specific myeloid cells, not common monocytes or macrophages. We named these precursors "cell cycle-arrested quiescent osteoclast precursors (QOP). QOP differentiate into osteoclasts without cell cycle progression. In the present study, I revealed that (1) QOP circulate in the blood and settle in the bone in response to bone resorption stimuli, (2) the expression level of RANK is up-regulated by M-CSF(CSF1) in the QOP along the bone surface, and the expression of c-Fos in the QOP is also necessary for the up-regulation of RANK.

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（金額単位：円）

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１．研究開始当初の背景

以前我々は、細胞増殖が停止した破骨細胞前駆細胞(Cell cycle-arrested quiescent osteoclast precursors: QOP)を同定した(J Cell Biol. 2009; 184:541-554)。これまでの研究成果より、生体内において、破骨細胞は QOP から分化することを示したが、QOP の生体内におけるキャラクターとその分化過程は明らかではない。

２．研究の目的

破骨細胞は QOP から分化する。以上より、

生体内におけるQOPの数および挙動が骨吸収に影響を及ぼすことが考えられる。生体内にはQOPプールが存在するという仮説を立て、生体内におけるQOPのキャラクター、および分化調節機構を調べることを目的とした。

３．研究の方法

(1) マウス骨髄からのQOPの分取

マウス骨髄細胞をRANKおよびFms抗体を用いてセルソーターにより分取した。分取した細胞において、ヒドロキシウレアの存在下、および非存在下でのM-CSFおよびRANKL誘導性の破骨細胞分化を調べた。ヒドロキシウレア存在下でも破骨細胞に分化する細胞画分をQOPとした。

(2)　マイクロアレイ解析

マウス骨髄細胞をセルソーターにより分取し、細胞抽出液からmRNAを調製した。cDNAを合成し、マイクロアレイ解析に用いた。

(3) 異所性骨形成実験系

リコンビナントhuman BMPをコラーゲンペレットに添加した。ペレットを野生型マウスおよびRANKL欠損マウスの背部筋膜下に移植した。2週後にペレットを摘出し、パラフィン切片および凍結切片を作成し、免疫組織解析に用いた。

４．研究成果

(1) 静止期破骨細前駆細胞(QOP)の分取とキャラクター解析

以前我々は、ヒドロキシウレアはマクロファージの破骨細胞分化を抑制するが、QOPの破骨細胞分化は抑制しないことを報告した(J Cell Biol. 2009; 184:541-554)。マウス骨髄細胞をRANKおよびFms抗体を用いて4つの分画に分けた。それぞれの画分をセルソーターにより分取し、ヒドロキシウレア存下における破骨細胞分化能を調べた。その結果、RANK(low) Fms(high)画分はヒドロキシウレアにより破骨細胞分化が抑制されたが、RANK(high) Fms(low)画分は抑制されなかった。(雑誌発表8, Fig. 1, b and c)。以上より、RANK(high) Fms(low)画分にQOPが含まれていることが明らかになった(以降QOP画分)。さらにQOP画分とRANK(low) Fms(high)画分のキャラクターを、FACSおよびマイクロアレイ解析にて調べた。その結果、QOP画分はRANK(low) Fms(high)画分に比較し、マクロファージマーカー(F4/80, CD11b, Fms)発現が低く、破骨細胞マーカー(Carbonic anhydrase II, MMP9, TRAP5b, Trasferrin receptor)発現が高いことを明らかにした。以上より、QOPはRANK(low) Fms(high)画分(以降マクロファージ画分)と比較し、破骨細胞分化へコミットした細胞であることが示唆された（雑誌発表8, Fig. 1, d and Table 1）。

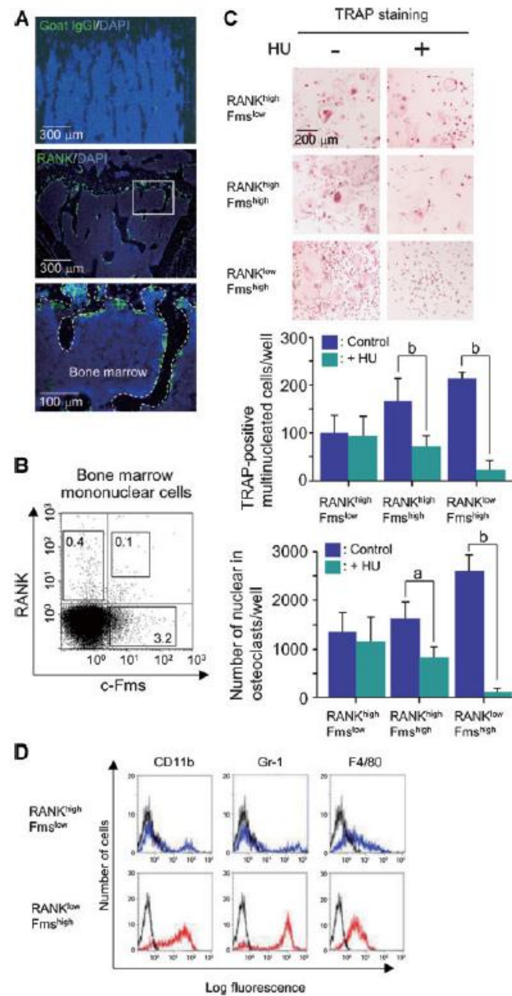


Fig. 1. Identification of QOPs in mouse bone marrow. (*A*) Tibial sections prepared from 6-week-old mice were stained for RANK (green) and DAPI (nuclei, blue) (middle panel). The lower panel shows an enlargement of the boxed area in the middle panel. Dotted lines indicate bone. Biotinylated goat IgG was used for the control of anti-RANK antibody. The upper panel shows the control for RANK staining. (*B*) Bone marrow cells obtained from tibiae were analyzed for the expression of RANK and c-Fms using FACS. Percentages of $RANK^{high}Fms^{low}$ cells, $RANK^{high}Fms^{high}$ cells, and $RANK^{low}Fms^{high}$ cells were provided in each square fraction. (*C*) $RANK^{high}Fms^{low}$, $RANK^{high}Fms^{high}$, and $RANK^{low}Fms^{high}$ cells were isolated from bone marrow and were cultured with M-CSF (10^4 units/mL) and RANKL (5 nM) in the presence or absence of hydroxyurea (50 μM) in 96-well plates (1×10^4 cells/well). After culturing for 5 days, cells were fixed and stained for TRAP (upper panels). TRAP-positive cells containing more than three nuclei were counted as osteoclasts (middle panel). The total number of nuclei in osteoclasts were counted (lower panel). Results are expressed as the mean ± SD for three cultures. Significantly different from control, $^a p < 0.05$, $^b p < 0.01$. (*D*) $RANK^{high}Fms^{low}$ cells (blue histogram) and $RANK^{low}Fms^{high}$ cells (red histogram) were analyzed for expression of CD11b, Gr-1, and F4/80 by FACS. Black histograms correspond to IgG isotype controls. Data are representative of three independent experiments.

Table 1. Relative Expression for Macrophage Markers and Osteoclast Markers in $RANK^{high}Fms^{low}$ Cells and $RANK^{low}Fms^{high}$ Cells

Gene description	Gene symbol	Fold change (normalized) $RANK^{high}Fms^{low}/RANK^{low}Fms^{high}$
Macrophage markers		
EGF-like module containing, mucin-like, hormone receptor-like sequence 1	Emr1 (F4/80)	0.3
Integrin alpha M	Itgam (CD11b)	0.6
Colony stimulating factor 1 receptor	Csf1r (c-Fms)	0.2
Osteoclast markers		
Carbonic anhydrase II	Car II	4.0
Matrix metallopeptidase 9	Mmp9	3.7
Acid phosphatase 5, tartrate-resistant	Acp5 (TRAP5b)	2.9
Transferrin receptor	Tfrc	2.6

$RANK^{high}Fms^{low}$ cells and $RANK^{low}Fms^{high}$ cells were isolated from bone marrow in mice by FACS. Differential expression levels of macrophage and osteoclast markers were determined by GeneChip analysis. The numbers were calculated by dividing the fold changes of genes in $RANK^{high}Fms^{low}$ cells by the fold changes of genes in $RANK^{low}Fms^{high}$ cells. EGF = epidermal growth factor; RANK = receptor activator of NF-κB.

QOP画分とマクロファージ画分の増殖能、貪食能、および樹状細胞への分化能を比較した。その結果、QOP画分はマクロファージ画分と比較し、増殖能、貪食能、および樹状細胞への分化能が低いことが明らかになった(雑誌

発表 8, Fig. 2)。

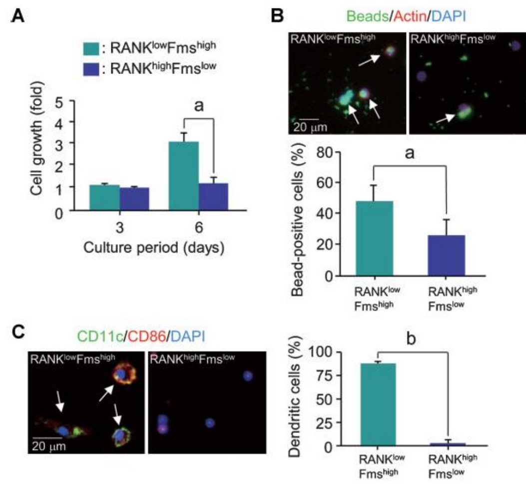


Fig. 2. Characterization of $RANK^{high}Fms^{low}$ cells and $RANK^{low}Fms^{high}$ cells in bone marrow. $RANK^{high}Fms^{low}$ cells and $RANK^{low}Fms^{high}$ cells were isolated from bone marrow using FACS. (*A*) Cell growth. $RANK^{high}Fms^{low}$ cells and $RANK^{low}Fms^{high}$ cells were cultured for 3 and 6 days in the presence of M-CSF (10^4 units/mL). Cell growth was measured by the Alamar Blue assay and expressed as the fold-increase over $RANK^{low}Fms^{high}$ cells at day 3. (*B*) Phagocytosis. $RANK^{high}Fms^{low}$ cells and $RANK^{low}Fms^{high}$ cells were cultured in serum-free medium for 4 hours in the presence of M-CSF (10^4 units/mL), and then incubated for 40 minutes with latex beads (green). Cells were stained for rhodamine-conjugated phalloidin (red) and DAPI (blue) (upper panels). Arrows indicate bead-positive cells. Cells incorporating more than 30 beads were counted as bead-positive cells (lower panel), and percentages of bead-positive cells were determined. (*C*) Dendritic cell differentiation. $RANK^{high}Fms^{low}$ cells and $RANK^{low}Fms^{high}$ cells were cultured for 3 days with GM-CSF (10 ng/mL), and further treated for 24 hours with GM-CSF (10 ng/mL) and lipopolysaccharide (1 μg/mL). Cells were stained for CD11c (green), CD86 (red), and DAPI (blue) (left panels). Arrows indicate CD11c and CD86 double-positive cells (yellow cells). Percentages of CD11c and CD86 double-positive ($CD11c^+CD86^+$) cells were determined (right panel). Results are expressed as the mean ± SD for three cultures. Significantly different from cultures of $RANK^{low}Fms^{high}$ cells, $^ap < 0.05$, $^bp < 0.01$.

（2）QOP の in vivo 解析-1

生体内における破骨細胞は細胞周期が停止した QOP から分化するか否かを BMP を用いた異所性骨形成実験により調べた。その結果、異所性骨中に形成される破骨細胞は（1）QOP から分化すること、（2）血流中の QOP が異所性骨に遊走することが明らかになった(雑誌発表 8, Fig. 3、4、5、6)。

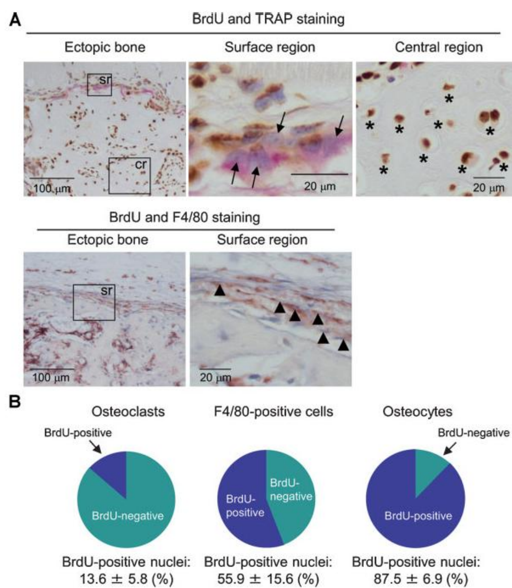


Fig. 3. Incorporation of BrdU into osteoclasts, osteocytes, and F4/80-positive cells in BMP-2–induced ectopic bone. BMP disks were implanted for 2 weeks into mice, which were given BrdU in drinking water (1 mg/mL). BMP-disks were then recovered and processed for tissue observation. (*A*) Sections of BMP disks were double-stained for TRAP (red) and BrdU (brown) (upper panels). Sections of BMP disks were double-stained for F4/80 (brown) and BrdU (blue) (lower panels). The surface region (sr) and central region (cr) of ectopic bones are indicated in squares in the left panels. The right panels show enlargement of the images of surface and central regions in the left panels. Arrows and asterisks indicate BrdU-negative nuclei in osteoclasts and BrdU-positive nuclei in osteocytes, respectively. Arrowheads indicate BrdU-positive nuclei in F4/80-positive cells. (*B*) BrdU-positive and -negative nuclei were counted and percentages of BrdU-positive nuclei were calculated. Results are expressed as the mean ± SD for three animals.

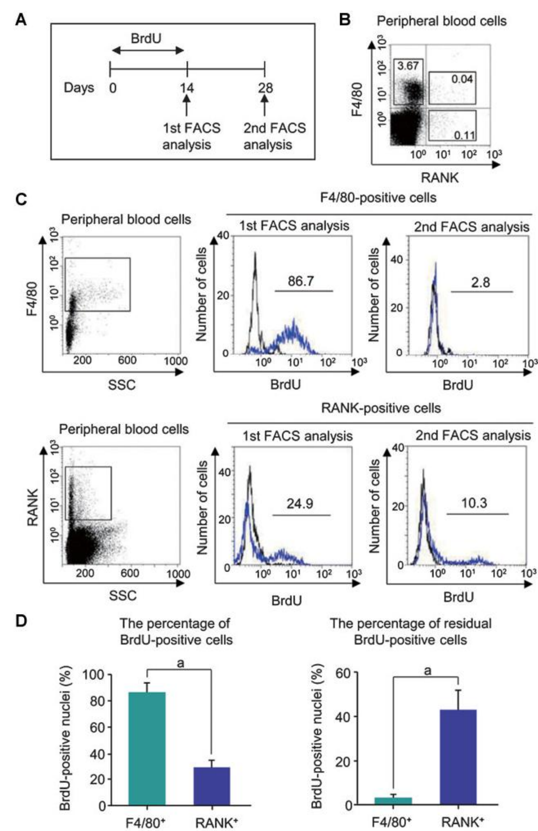


Fig. 4. Incorporation of BrdU into nuclei of F4/80-positive cells and RANK-positive cells in peripheral blood. (*A*) The experimental protocol. One group of mice was given BrdU in drinking water (1 mg/mL) for 2 weeks. The other group was given BrdU in drinking water for 2 weeks, and further maintained for 2 weeks without BrdU. Mice were euthanized to collect peripheral blood. (*B*) Peripheral blood mononuclear cells were analyzed for the expression of RANK and F4/80 using FACS. Percentages of $F4/80^{high}RANK^{low}$ cells, $F4/80^{high}RANK^{high}$ cells, and $F4/80^{low}RANK^{high}$ cells were provided in each square fraction. (*C*) F4/80-positive cells and RANK-positive cells were gated (left panels). The incorporation of BrdU into F4/80-positive cells (upper panels) and RANK-positive cells (lower panels) was analyzed by FACS. Blue and black histograms indicate the results obtained using anti-BrdU antibodies and IgG isotype controls, respectively. Percentages of BrdU-positive cells are provided in each figure. Data are representative of three independent experiments. (*D*) Percentages of BrdU-positive cells among F4/80-positive cells and RANK-positive cells were determined in the first FACS analysis (left panel). The percentage of residual BrdU-positive cells was calculated by dividing the percentage of BrdU-positive cells in the second FACS analysis by that in the first FACS analysis (right panel). Results are expressed as the mean ± SD for three experiments. Significantly different from $F4/80^+$, $^ap < 0.01$.

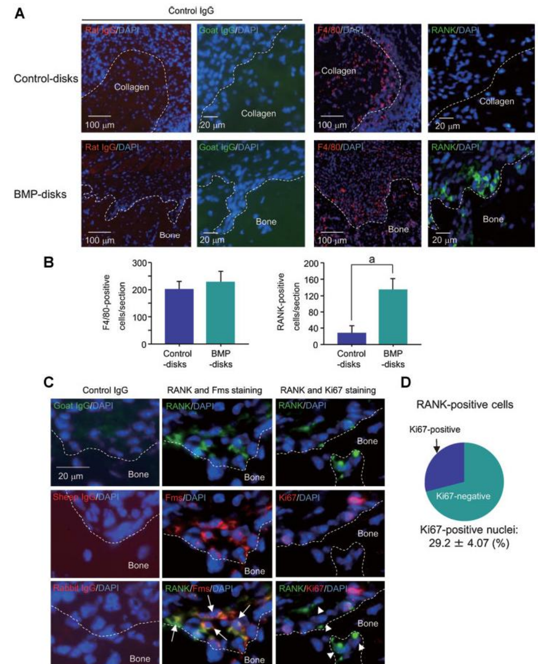


Fig. 5. Identification of QOPs in BMP-disks in RANKL$^{-/-}$ mice. BMP disks and control disks were implanted into RANKL$^{-/-}$ mice, and recovered 2 weeks later. (*A*) Sections of the BMP disks and control disks were stained for F4/80 (red) or RANK (green). Biotinylated goat IgG and rat IgG were used for the control of anti-RANK and anti-Ki67 antibodies, respectively. Nuclei were detected by DAPI staining (blue). (*B*) Numbers of F4/80-positive cells (left) and RANK-positive cells (right) were counted. Results are expressed as the mean ± SD for three experiments. Significantly different from control-disks, $^{a}p < 0.01$. (*C*) Sections of the BMP-disks were subjected to double staining of RANK (green) and c-Fms (red) (middle), or to double staining of RANK (green) and Ki67 (red) (right). Nuclei were detected by DAPI staining (blue). The arrows in the middle panel indicate mononuclear cells double-positive for RANK and c-Fms (yellow cells). The arrowheads in the right panel indicate RANK-positive and Ki67-negative cells. Biotinylated goat IgG, sheep IgG, and rabbit IgG were used for the control of anti-RANK, anti-Fms, and anti-Ki67 antibodies, respectively. (*D*) Ki67-positive and Ki67-negative nuclei in RANK-positive cells were counted and percentages of Ki67-positive nuclei were calculated. Results are expressed as the mean ± SD for three BMP disks.

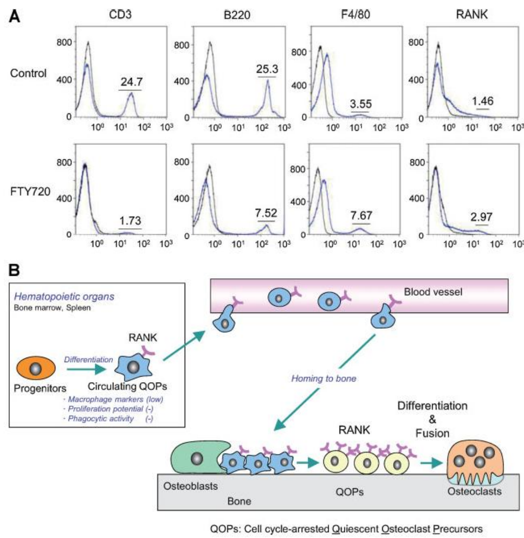
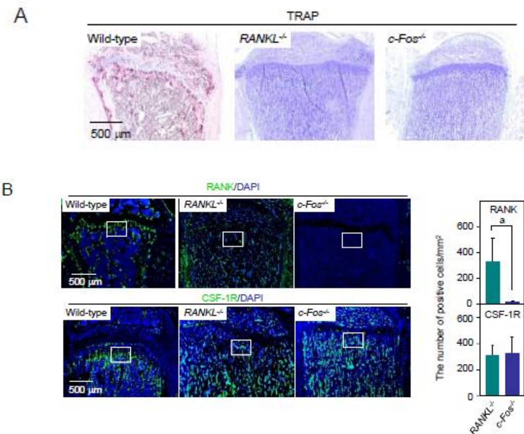


Fig. 6. Effect of FYT720 on peripheral QOPs in mice. (*A*) Eight-week-old mice were injected with FYT720 (3 mg/kg body weight) or vehicle (control). Five hours later, mice were sacrificed. Peripheral blood cells were collected, and analyzed for the expression of CD3e, B220, F4/80, and RANK by using FACS. Data show the representative FACS profiles. Similar profiles of blood cells were obtained in the two additional independent experiments. (*B*) Schematic diagram of osteoclastogenesis in vivo. Lineage-committed QOPs are generated in hematopoietic organs. Some QOPs enter the bloodstream and home to the correct location for osteoclastogenesis. RANK expression of QOPs is upregulated there. QOPs fuse each other, and differentiate into osteoclasts in response to bone-resorbing stimuli without cell-cycle progression. Osteoblasts may play an essential role in the homing of QOPs to bone.

(3) QOP の in vivo 解析-2

骨硬化症モデルマウスの骨組織におけるRANK 陽性細胞の局在を調べた。その結果、野生型マウス、および RANKL 欠損マウスの骨表面には多数の RANK 陽性細胞が認められた。一方、c-Fos 欠損マウスの骨表面には RANK 陽性細胞は全く認められなかった(雑誌発表 1, Fig. 1)。一方、CSF1R 陽性マクロファージの発現は全てのマウスにおいて認められた。以上の結果より、骨表面における破骨細胞前駆細胞の RANK の発現上昇には c-Fos が必要であることが明らかになった。



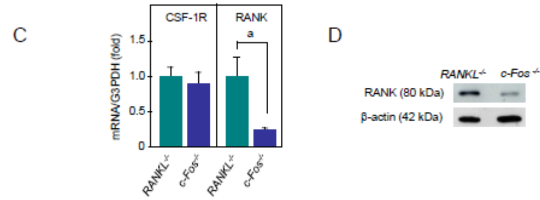


Figure 1. Distribution of RANK-positive cells and CSF-1R-positive cells in bone in wild-type mice, *RANKL*$^{-/-}$ mice and *c-Fos*$^{-/-}$ mice.

(A, B) Sections of tibiae were prepared from 6-week-old wild-type mice, *RANKL*$^{-/-}$ mice, and *c-Fos*$^{-/-}$ mice. (A) Sections were stained for TRAP. TRAP-positive cells appeared red. (B) Sections were stained for RANK (green, upper panels), and CSF-1R (green, lower panels). Nuclei were detected by DAPI staining (blue). Numbers of RANK-positive cells and CSF-1R-positive cells in 0.135 mm^{2} of the central area just under the growth plate (rectangles) were counted in three images prepared from three *RANKL*$^{-/-}$ mice and *c-Fos*$^{-/-}$ mice (right panel). Results are expressed as the mean ± s.d. for three images. $^{a}p<0.01$. The representative image was shown in the left panel. (C) Total RNA was extracted from tibiae of *RANKL*$^{-/-}$ mice and *c-Fos*$^{-/-}$ mice. Expression levels of RANK and CSF-1R mRNAs were estimated by quantitative real-time RT-PCR. Results are expressed relative to the levels in *RANKL*$^{-/-}$ mice. Results are expressed as the mean ± s.d. for three mice. $^{a}p<0.01$. (D) Bone lysates were prepared from tibiae of *RANKL*$^{-/-}$ mice and *c-Fos*$^{-/-}$ mice, and subjected to Western blot analysis using anti-RANK antibody.

次に、脾臓マクロファージにおける RANK の発現を調べた。その結果、骨髄と同様に、脾臓においても c-Fos 欠損マウスでは RANK の発現が認められなかった。一方、胸腺およびパイエル板における RANK の発現は全てのマウスにおいて認められた(雑誌発表 1, Fig. 2)。

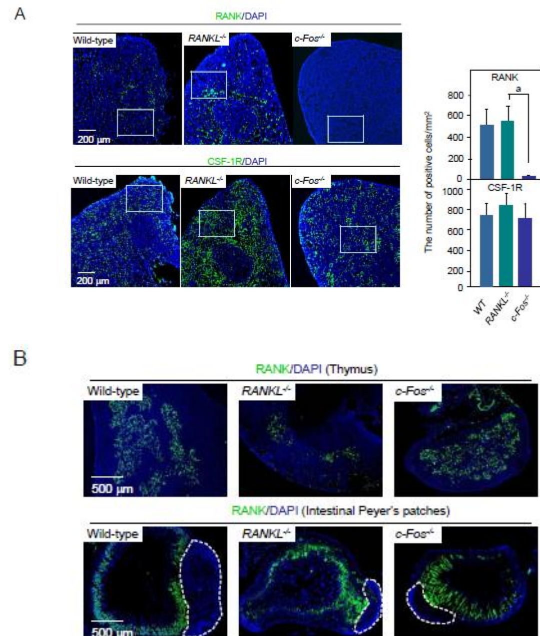


Figure 2. Distribution of RANK-positive cells in spleen, thymus and intestinal Peyer's patches in wild-type mice, *RANKL*$^{-/-}$ mice and *c-Fos*$^{-/-}$ mice.

(A) Sections of spleen were prepared from 6-week-old wild-type mice, *RANKL*$^{-/-}$ mice, and *c-Fos*$^{-/-}$ mice, and stained for RANK (green, upper panels), and CSF-1R (green, lower panels). Nuclei were detected by DAPI staining (blue). Numbers of

RANK-positive cells and CSF-1R-positive cells in 0.135 mm^2 of the red pulp region (rectangles) were counted in three images prepared from three wild-type mice, *RANKL*$^{-/-}$ mice, and *c-Fos*$^{-/-}$ mice (right panel). Results are expressed as the mean ± s.d. for three images. $^a p<0.01$. The representative image was shown in the left panel. (B) Sections of thymus and intestinal Peyer's patches were prepared from 6-week-old wild-type mice, *RANKL*$^{-/-}$ mice, and *c-Fos*$^{-/-}$ mice. Sections were stained for RANK (green). Nuclei were detected by DAPI staining (blue). Peyer's patches are indicated by dashed circles in lower panels.

c-Fos 欠損マウスの心臓から野生型マウス由来の骨髄細胞を移植すると、骨表面に RANK 陽性細胞が出現した(雑誌発表 1, Fig. 3,a)。さらに in vitro 培養系にて、マクロファージによる RANK の発現上昇には(1) c-Fos が必要であること、(2) 骨芽細胞が発現する CSF1 が必要であることを明らかにした(雑誌発表 1, Fig. 3,b, c, d, Fig. 4)。

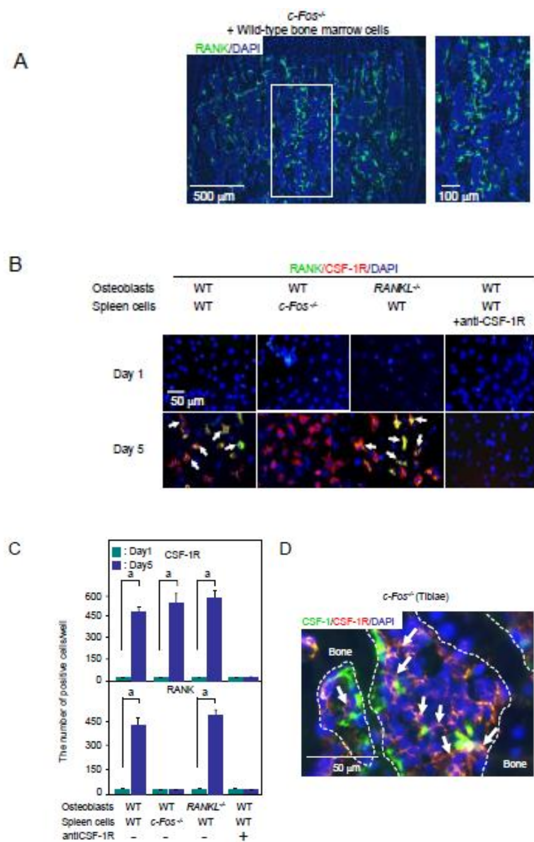


Figure 3. A bone environment is required for the up-regulation of RANK expression in osteoclast precursors.

(A) Wild-type mouse bone marrow cells were injected into the left cardiac ventricle of *c-Fos*$^{-/-}$ mice myelosuppressed with busulfan. After 18 days, sections of tibiae were prepared and stained for RANK (green). Nuclei were detected by DAPI staining (blue). Right panel, a high power view of the portion indicated. (B) Primary osteoblasts were prepared from calvariae of wild-type mice and *RANKL*$^{-/-}$ mice. Osteoblasts were cocultured for 1 day (upper panels) or 5 days (lower panels) with wild-type spleen cells or *c-Fos*$^{-/-}$ spleen cells. Anti-CSF-1R antibody (AFS98) was also added to some cocultures of wild-type osteoblasts and wild-type spleen cells. Cells were fixed and double-stained for RANK (green) and CSF-1R (red). Nuclei were detected by DAPI staining (blue). Arrows indicate cells double positive for CSF-1R and RANK (yellow cells). The representative image of three independent experiments was shown. (C) Numbers of RANK-positive cells (green, yellow) and CSF-1R-positive cells (red, yellow) shown in (B) were counted. Results are expressed as the mean ± s.d. for three cultures. $^a p<0.01$. (D) Tibiae were recovered from 6-week-old *c-Fos*$^{-/-}$ mice. Sections of tibiae were prepared and subjected to double staining of CSF-1 (Green) and CSF-1R (red). Nuclei were detected by DAPI staining (blue). Arrows indicate CSF-1R-positive cells which are in contact with CSF-1-expressing osteoblastic cells.

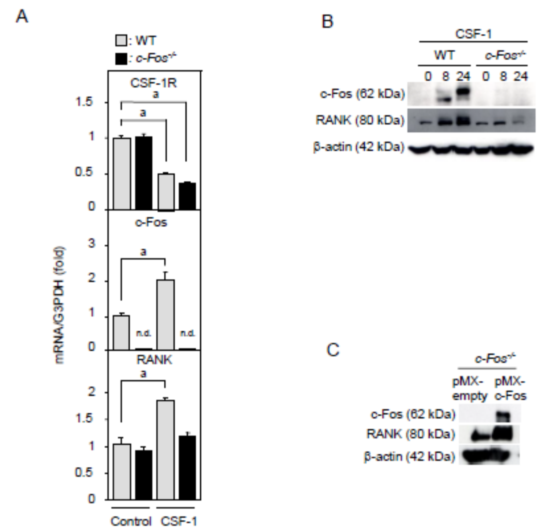


Figure 4. CSF-1 up-regulates RANK expression in osteoclast precursors.

(A) Spleen cells obtained form wild-type mice and *c-Fos*$^{-/-}$ mice were cultured for 2 days in the presence of CSF-1 (10^4 units/ml) to prepare macrophages. Splenic macrophages were further cultured in the absence of CSF-1 for 16 hours. Then cells were treated for 0 and 8 hours with CSF-1 (10^4 units/ml), and total cellular RNA was prepared. Levels of CSF-1R, c-Fos, and RANK mRNAs were estimated by quantitative real-time RT-PCR. Results are expressed relative to levels in the wild-type macrophages at 0 hour (control). Results are expressed as the mean ± s.d. for three cultures. $^a p<0.01$. n.d.: not detectable. (B) Wild-type and *c-Fos*$^{-/-}$ spleen macrophages were cultured for 0, 8, and 24 hours in the presence of CSF-1 (10^4 units/ml). Cell lysates were then prepared, and subjected to Western blot analysis using anti-c-Fos antibody and anti-RANK antibody. (C) Spleen macrophages were prepared from *c-Fos*$^{-/-}$ mice, and infected with empty pMX retrovirus (pMX-empty) or pMX retrovirus expressing c-Fos (pMX-c-Fos). Infected macrophages were cultured with CSF-1 (10^4 units/ml) for 48 hours, and cell lysates were prepared and subjected to Western blot analysis using anti-c-Fos and anti-RANK antibodies.

c-Fos 欠損マウス由来のマクロファージに RANK を過剰発現させ、M-CSF と RANKL による

破骨細胞分化への影響を調べた。その結果、RANK の過剰発現により c-Fos 欠損マウスの破骨細胞分化のレスキューは認められなかった(雑誌発表 1, Fig.5)。以上より、c-Fos は破骨細胞分化において、RANK の発現上昇および RANK の下流の両方に必要であることが明らかになった。

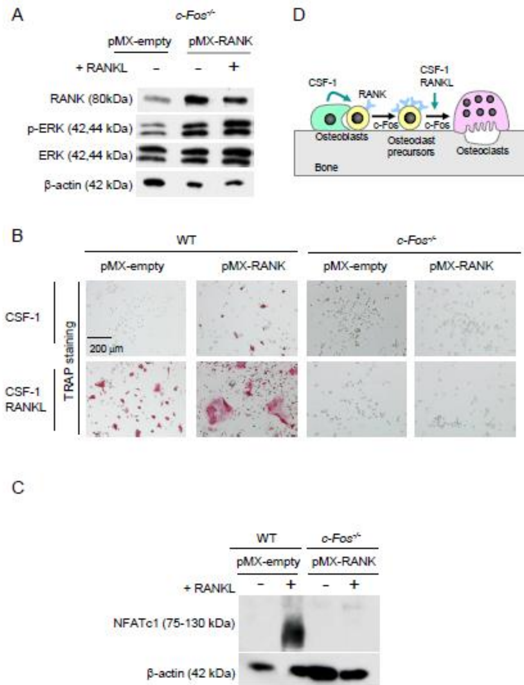


Figure 5. Overexpression of RANK in *c-Fos*-/- macrophages failed to induce their osteoclastic differentiation.

(A) *c-Fos*-/- spleen macrophages were infected with empty pMX retrovirus (pMX-empty) or pMX retrovirus expressing RANK (pMX-RANK), and incubated with RANKL for 0 and 15 min. Then, cell lysates were prepared and subjected to Western blot analysis using anti-RANK, anti-ERK, and anti-phosphorylated ERK antibodies. (B) Wild-type and *c-Fos*-/- spleen macrophages were infected with pMX-empty or pMX-RANK. Infected macrophages were cultured for 3 days with CSF-1 (10^4 units/ml) in the presence or absence of RANKL (5 nM). Cells were then fixed and stained for TRAP. (C) Wild-type and *c-Fos*-/- spleen macrophages were infected with pMX-empty and pMX-RANK, respectively. Infected macrophages were cultured for 3 days with CSF-1 (10^4 units/ml) in the presence or absence of RANKL (5 nM). Then cell lysates were prepared and subjected to Western blot analysis using anti-NFATc1 and β-actin antibodies. (D) A schematic model of osteoclastogenesis along the bone surface. The expression of RANK in osteoclast precursors is up-regulated by factors in the bone environment such as CSF-1 produced by osteoblasts in a c-Fos-dependent manner. Osteoclast precursors which express high levels of RANK differentiate into osteoclasts in response to RANKL and CSF-1. This differentiation process also requires c-Fos as an essential transcription factor.

５．主な発表論文等
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６．研究組織

(1)研究代表者

溝口　利英（MIZOGUCHI TOSHIHIDE）
松本歯科大学・総合歯科医学研究所・講師
研究者番号：９０３２９４７５